CELL SUSCEPTIBILITY TO TRANSFORMATION AND CYTOTOXICITY BY THE CARCINOGENIC HYDROCARBON BENZO[A]PYRENE

By Eliezer Huberman and Leo Sachs

SECTION OF GENETICS, WEIZMANN INSTITUTE OF SCIENCE, REHOVOTH, ISRAEL

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It has previously been shown that carcinogenic hydrocarbons can directly induce *in vitro* the transformation of normal hamster cells to tumor cells.[1, 2] The transformed cells showed a hereditary random pattern of cell growth and the ability to grow continuously in culture, and formed progressively growing tumors after subcutaneous inoculation into adult hamsters. Transformation in culture was obtained with four hydrocarbons that are carcinogenic *in vivo*, but not with three hydrocarbons reported as noncarcinogenic *in vivo*.[2] The present experiments with hamster embryo cells and the carcinogenic hydrocarbon benzo[a]pyrene (BP) were undertaken to determine (1) whether there is a "one-hit" dose response curve for transformation; (2) whether transformation and cytotoxicity by BP are two different events; (3) whether there are normal hamster cells that are resistant to the cytotoxic action of BP; (4) whether there is a hereditary and/or physiological state of competence to transformation, and (5) since there are BP-transformed colonies with different morphologies, whether the morphology of the transformed colony is determined by the morphology of the normal cells before transformation. Transformed colonies were identified by their random pattern of cell growth, and as in previous experiments,[1, 2] such colonies were not found in the untreated control cultures.

Materials and Methods.—Cell cultures: Primary or secondary cultures from minced whole embryos of golden hamsters served as a source of normal cells. These will be referred to as mixed embryo cells. Mass cultures of primaries were seeded at 1×10^7 cells per 100-mm Petri dish (Falcon Co.), and mass cultures of secondaries at 5×10^6 per 100-mm Petri dish. The cells were grown in Eagle's medium with a fourfold concentration of amino acids and vitamins (EM) with 10% calf serum.

Benzo[a]pyrene (BP): BP (10 mg) was dissolved in 1 ml acetone, and this BP acetone solution was suspended in 100 ml EM with 10% calf serum, warmed to 37°C, to make a stock solution. Stock solutions were stored at 4°C in the dark, and used from 2 to 14 days after they were made. The BP solutions were made and added to the cultures in a room with blacked-out windows which allowed the entrance of only a small amount of diffuse light necessary for visibility.

Clone isolations: Clones of normal hamster cells were isolated by the cylinder technique[3] and after seeding in soft agar.[4] In the cylinder technique, 5×10^2 to 2×10^3 primary cells were seeded on X-irradiated (4000 r) rat-embryo feeder layers,[5] and 6–8 days later, individual clones were isolated by placing over each clone a Pyrex cylinder whose lower rim had been coated with silicone grease. The cells were detached in the cylinder with 0.1–0.2 ml of a 0.05% trypsin and 0.1% versene solution, and the cells from each clone were transferred into a 50-mm Petri dish with an X-irradiated rat-cell feeder layer and EM with 10% fetal calf serum. For the isolation from soft agar, 2×10^5 cells from secondary cultures were seeded per 50-mm Petri dish in an upper layer of 0.3% agar in EM with 10% calf or fetal calf serum, on a 0.5% agar-medium base layer. Up to five colonies per plate, that were not composed of blood cells,[6] were found after 7–10 days, and each colony was then isolated with a pipette and transferred into a 50-mm Petri dish with an X-irradiated rat feeder layer and EM with 10% fetal calf serum. The cells within each colony isolated by this procedure and used in the present experiments had the same morphology, so that these normal colonies are presumably clones. The clones isolated by the cylinder technique were used for experiments without further passaging at 5–8 days after isolation; those isolated from the soft agar were passaged once at 8 days after isolation and then used for experiments 4 days later.

Out of 270 clones isolated by the cylinder technique, 180 grew after isolation, and of these, 44 formed a sufficient number of cells that nearly filled the Petri dish. For the transformation assay with BP, 27 of the 44 were re-cloned, and of these, 13 formed colonies of which ten could be scored in this assay. From the soft agar, 218 clones were isolated; 153 grew after isolation, and of these 48 formed a sufficient number of cells that nearly filled the Petri dish. For the transformation assay with BP, 20 of these 48 were re-cloned, and of these, 11 formed colonies that could be scored in this assay. Seventeen clones isolated by the cylinder technique and 18 clones isolated from soft agar were passaged further without treatment with BP, and all degenerated after 3–6 weeks in culture. These normal isolated hamster clones thus showed the limited life span in culture found in previous experiments with other cultures of normal hamster cells.[2, 7]

Transformation assay: Transformation by BP has been obtained after treatment of normal hamster cells cloned either with or without a feeder layer of X-irradiated rat embryo cells. Since the use of these feeder layers gave a higher cloning efficiency, cells in the transformation assay were cloned on X-irradiated rat feeder layers. Mixed embryo cells were seeded at 2–5 $\times$ 10^3 cells per 50-mm Petri dish in EM with 10% calf serum, and isolated clones at 1–10 $\times$ 10^3 cells per 50-mm Petri dish in EM with 10% fetal calf serum. BP was added 1 day after the cells were seeded for the assay, and the plates were fixed in methanol and stained with May-Grünwald-Giemsa at 9 or 10 days after seeding.[2, 5] There was no medium change during this 9- or 10-day period, so that the BP was present from 1 day after seeding until the plates were stained. The total number of colonies and the number of transformed colonies was always obtained by a direct count in plates that did not contain totally confluent colonies. The per cent of transformed colonies is given as a per cent of the total number of colonies.

Results.—Dose response curve for transformation and cytotoxicity in mixed embryo cells: When mixed embryo cells were treated with different concentrations of BP, the per cent of transformed colonies was directly related to the dose of carcinogen up to 10 μg BP/ml. The dose response curve for transformation was linear up to this concentration of BP, with a slope of 0.8 on a log-log plot (Fig. 1), and this indicates that there is a "one-hit" response for transformation by this carcinogen.

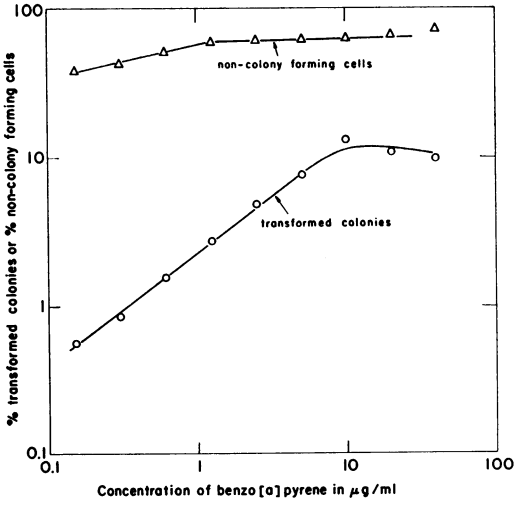

FIG. 1.—Dose response curve for transformation and cytotoxicity by BP after treatment of mixed hamster embryo cells. The difference between the number of colonies in the control and in the BP-treated cultures is referred to as non-colony-forming cells. Cytotoxicity is expressed in the form of non-colony forming cells as a per cent of the number of colonies in the control. The carcinogen was added 1 day after cells were plated for cloning, and colonies were fixed and stained at 9 or 10 days after plating. Average results of three expts.

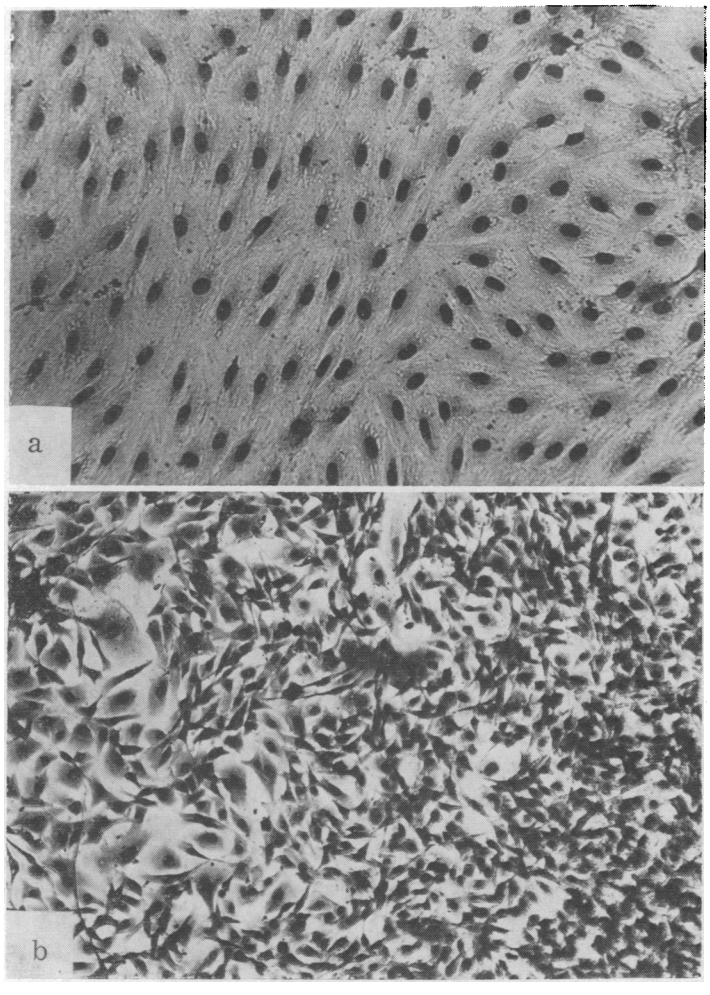

FIG. 2.—(*a*) Normal cell colony. (*b*) Dense BP-transformed colony (5 μg BP/ml). The colonies were fixed and stained 10 days after the cells were plated for cloning. ×80.

Transformed colonies with four different morphologies were observed after treatment of these mixed embryo cells, one type giving denser transformed colonies (Fig. 2) than the other types. A count of only the dense transformed colonies gave the same slope for the dose response curve for transformation as a count of all four types of transformed colonies (Fig. 3 and Table 1).

A comparison of the dose response curve for transformation with that for cytotoxicity indicates that the two curves are different, and that the per cent of transformed colonies continued to increase linearly after the curve for cytotoxicity had reached a plateau (Fig. 1 and Table 1). It is also of interest that there were nontransformed colonies after treatment with 40 μg/ml BP. These results on the treatment of mixed embryo cells thus indicate that transformation and cytotoxicity by BP are two different events, and that there were normal cells that were resistant to cytotoxicity and either susceptible or resistant to transformation.

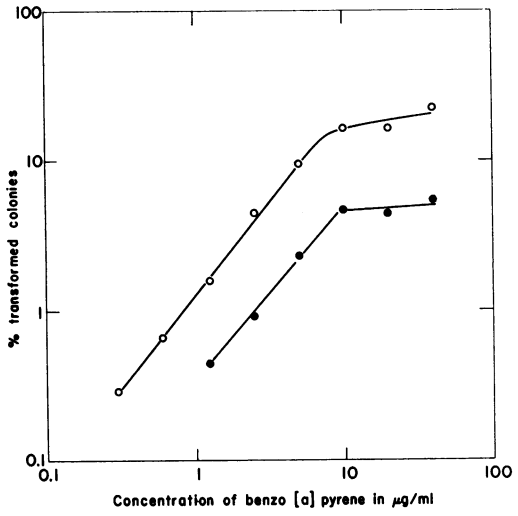

FIG. 3.—Dose response curve for transformation by BP after treatment of mixed hamster embryo cells. (●) Dense transformed colonies. (○) All morphological types of transformed colonies. The carcinogen was added 1 day after cells were plated for cloning, and colonies were fixed and stained at 10 days after plating.

Transformation and cytotoxicity in isolated embryo cell clones: Experiments with isolated clones were carried out in order to clarify further the cellular basis for transformation and cytotoxicity by BP, and to determine whether there is a hereditary and/or physiological state of competence to transformation. The analysis of 21 isolated clones has indicated that the normal cell clones can be divided into those that were resistant and those with varying degrees of susceptibility to cytotoxicity by BP; that transformation was obtained after treatment of both types of clones; and that there were clones with no transformation. The results from clones with transformation are shown in Table 2, and those with no transformation in Table 3. Seven of the 21 clones tested were resistant to cytotoxicity at the BP concentrations tested, and this per cent of resistant clones is of about the same order of magnitude as the per cent of resistant colonies after treatment of mixed embryo cells with similar concentrations of BP (Table 1).

TABLE 1

PER CENT CLONING EFFICIENCY AND PER CENT TRANSFORMED COLONIES AFTER TREATMENT OF MIXED HAMSTER EMBRYO CELLS WITH BP

Conc. of BP (μg/ml)	Total no. of colonies	Cloning efficiency (%)	No. Transformed Colonies		Transformed Colonies (%)	
			Total	Dense	Total	Dense
0	731	2.9	0	0	0	0
0.3	688	2.0	2	0	0.3	0
0.6	454	1.5	3	0	0.7	0
1.2	441	1.3	7	2	1.6	0.5
2.5	756	1.0	36	7	4.8	0.9
5	777	1.1	74	18	9.5	2.3
10	877	0.9	147	41	16.8	4.8
20	539	0.7	88	24	16.3	4.5
40	164	0.5	37	9	22.6	5.5

BP was added 1 day after cells were plated for cloning, and the colonies were fixed and stained at 10 days after plating.

TABLE 2

PER CENT CLONING EFFICIENCY AND PER CENT TRANSFORMED COLONIES AFTER TREATMENT OF ISOLATED HAMSTER EMBRYO CELL CLONES WITH BP: CLONES WITH TRANSFORMATION

Clone no.	Conc. of BP (μg/ml)	Total no. of colonies	Cloning efficiency (%)	Transformed colonies (%)
1	0	306	1.2	0
	1	203	0.7	7.8
	10	232	0.8	60.8
2	0	600	3.7	0
	0.05	339	2.1	0.6
	0.5	79	0.5	2.5
	5	0	0	0
3	0	672	1.7	0
	1	237	0.8	3.4
	10	361	0.9	23.3
4	0	173	0.4	0
	1	128	0.4	0.8
	10	109	0.3	15.6
5	0	674	5.6	0
	5	739	3.2	4.5
6	0	322	0.8	0
	1	211	0.5	0.5
	10	256	0.7	2.0
7	0	148	0.7	0
	1	153	0.3	0
	10	82	0.3	1.2
8	0	832	2.7	0
	1	618	1.5	0.2
	10	600	1.5	0.7

Clone nos. 1, 3, 4, 6, and 8 were isolated from agar, and nos. 2, 5, and 7 by the cylinder technique. Further details on these clones are given in the legend to Table 3. BP was added 1 day after cells were plated for cloning, and the colonies were fixed and stained at 9 or 10 days after plating.

The existence of clones with no transformation and of clones with from 0.7 per cent to 60.8 per cent transformed colonies indicates that there was both a hereditary and a physiological state of cell competence to transformation. In contrast to the four morphological types of transformed colonies obtained after BP treatment of mixed embryo cells, each isolated clone with transformation gave transformed colonies with only one type of morphology. Spontaneous transformation was not observed in any of these 21 clones, nor in 35 other clones isolated as described in *Materials and Methods* and passaged without BP treatment.

Discussion.—The present results have indicated that there is a "one-hit" dose response curve for transformation by the carcinogenic hydrocarbon BP, as with transformation induced by polyoma virus.[4] The observation of transformed colonies with only one morphology after BP treatment of isolated clones has also been found for transformation by polyoma,[8] so that in both cases the morphology of the normal cell type that is transformed is a major factor in controlling the morphology of the transformed colony. For a further comparison between these two transforming agents, it is of interest that the results indicate a higher degree of hereditary heterogeneity for suceptibility to transformation of hamster embryo cells by BP than has been found with polyoma,[5, 8] and that there is a physiological state of competence to transformation with both agents. Whether the physiological state required for transformation is the same for BP and for polyoma remains to be determined.

TABLE 3

PER CENT CLONING EFFICIENCY AFTER TREATMENT OF ISOLATED HAMSTER EMBRYO CELL CLONES WITH BP: CLONES WITH NO TRANSFORMATION

Clone no.	Conc. of BP (μg/ml)	Total no. of colonies	Cloning efficiency (%)	Clone no.	Conc. of BP (μg/ml)	Total no. of colonies	Cloning efficiency (%)
9	0	510	2.0	16	0	434	1.2
	5	768	1.9		1	0	0
	25	738	1.9		10	0	0
10	0	590	1.5	17	0	844	2.1
	5	606	1.5		1	36	0.1
	25	640	1.6		10	45	0.1
11	0	647	1.9	18	0	335	0.8
	5	1172	1.8		5	0	0
12	0	85	0.2	19	0	406	5.8
	1	48	0.2		5	13	0.01
	10	96	0.2		25	3	0.02
13	0	639	1.6	20	0	226	1.3
	1	497	1.2		1	9	0.6
	10	614	1.5		10	0	0
14	0	391	1.0	21	0	254	1.0
	1	215	0.5		1	18	0.03
	10	215	0.5		10	5	0.01
15	0	573	1.4				
	1	448	1.2				
	10	423	1.1				

Clone nos. 12–17 were isolated from agar, and nos. 9–11 and 18–21 by the cylinder technique. The clones in this table and in Table 2 were tested for BP-induced transformation in six separate expts. Clone nos. 1, 4, and 6 in expt. I; 5 in expt. II; 2, 9, and 10 in expt. III; 11, 18, and 19 in expt. IV; 7, 20, and 21 in expt. V; and 3, 8, and 12–17 in expt. VI. At the end of the expts. the cells had been in culture for 29 days (expt. I), 20 days (expt. II), 23 days (expts. III and IV), 25 days (expt. V), and 28 days (expt. VI). BP was added 1 day after cells were plated for cloning, and the colonies were fixed and stained at 9 or 10 days after plating.

The results have also indicated that transformation and cytotoxicity by BP are two different events, that there are normal cells resistant to cytotoxicity by BP, and that transformation can be obtained after BP treatment of normal cells that are either resistant or susceptible to cytotoxicity. It will be of interest to use normal cells resistant or susceptible to cytotoxicity or to transformation, and to determine to what extent the binding of carcinogenic hydrocarbons to cellular constituents, including DNA,[9] is related to the cytotoxic and/or the transforming property of the hydrocarbon. The observation that there are normal cells resistant to cytotoxicity by BP also raises the possibility, which is now being further studied, that the resistance to cytotoxicity by carcinogenic hydrocarbons observed with different types of transformed cells[1, 2, 10] may be due to the selection of cells that were resistant as normal cells, rather than to the acquisition of resistance as the result of transformation.

Summary.—It has been shown that there is a "one-hit" dose response curve for transformation by the carcinogenic hydrocarbon benzo[a] pyrene (BP), and that transformation and cytotoxicity by BP are two different events. Results obtained with isolated clones indicate that normal hamster clones can be either resistant or susceptible to cytotoxicity by BP; that transformation can be obtained with both types of clones; that there is a hereditary and a physiological state of competence to transformation; and that the morphology of the normal cell type that is transformed is a major factor in controlling the morphology of the transformed colony.

[1] Berwald, Y., and L. Sachs, *Nature*, **200**, 1182 (1963).

[2] Berwald, Y., and L. Sachs, *J. Natl. Cancer Inst.*, **35**, 641 (1965).

[3] Puck, T. T., P. I. Marcus, and S. J. Cieciura, *J. Exptl. Med.*, **103**, 273 (1965).

[4] MacPherson, I., and L. Montagnier, *Virology*, **23**, 291 (1964).

[5] Sachs, L., D. Medina, and Y. Berwald, *Virology*, **17**, 491 (1962).

[6] Pluznik, D., and L. Sachs, *J. Cellular Comp. Physiol.*, **66**, 199 (1965); Ichikawa, Y., D. Pluznik, and L. Sachs, these PROCEEDINGS, **56**, 488 (1966).

[7] Borek, C., and L. Sachs, *Nature*, **210**, 276 (1966).

[8] Medina, D., and L. Sachs, *Virology*, **27**, 398 (1965).

[9] Brookes, P., and P. D. Lawley, *Nature*, **202**, 781 (1964).

[10] Haddow, A., *J. Pathol. Bacteriol.*, **47**, 581 (1938); Vasiliev, J. M., and V. I. Guelstein, *J. Natl. Cancer Inst.*, **31**, 1123 (1963); Alfred, L. J., A. Globerson, Y. Berwald, and R. T. Prehn, *Brit. J. Cancer*, **18**, 159 (1964).